

Detection of Lipocortin 1 in Human Lung Lavage Fluid: Lipocortin Degradation As a Possible Proteolytic Mechanism in the Control of Inflammatory Mediators and Inflammation

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Lipocortins are structurally related, glucocorticoid-inducible proteins that inhibit phospholipase A₂ (PLA₂), thereby reducing the liberation of arachidonic acid from phospholipids and so limiting the synthesis of eicosanoid inflammatory mediators. This study is the first demonstration of one lipocortin, lipocortin 1 (Lc 1; 37 kDa), in human lung lavage supernatants. In lavage fluid from healthy volunteers, a higher percentage (> 70%) of the detected Lc 1 was in its native form, compared to that from patients with abnormal lungs. In patients' lavage fluids, Lc 1 was more likely to be partially degraded (34 kDa). In abnormal bronchoalveolar lavage fluid (BALF), the more polymorphonuclear neutrophils (PMN)/lavage, the lower the proportion of Lc 1 in the native (37 kDa) form ($n = 7$ pairs, $rs = -0.8214$, $p < 0.05$). Furthermore, when BALF cells were cultured and the harvested conditioned media incubated with pure human recombinant Lc 1, degradation of the 37 kDa form increased with the percentage of PMN ($n = 10$ pairs, $s = -0.7200$ after 1 hr; $n = 6$ pairs, $rs = -0.9241$ after 6 hr). These results suggest that factors released from the PMN are responsible for Lc 1 degradation in man. When recombinant human Lc 1 was incubated with human neutrophil elastase, the enzyme degraded Lc 1 in a dose-dependent way, suggesting that neutrophil elastase may be one such factor. Since PMNs are ubiquitous at sites of inflammation, it is possible that Lc 1 degradation is a permissive mechanism, which ensures that sufficient inflammation occurs to destroy the provocative stimulus. However, it is equally possible that, in some circumstances, the mechanism may be pathological and that the inactivation of Lc 1 leads to chronic, uncontrolled inflammation.

Introduction

Although glucocorticoids have been used empirically in the treatment of inflammatory diseases for 40 years, their mechanisms of action are only now being elucidated. Studies in animals show that the anti-inflammatory actions of steroids can be mediated, at least in part, by a family of structurally related proteins named lipocortins 1 through 6. These anti-inflammatory proteins, which are synthesized in response to both naturally occurring and synthetic glucocorticoids (1,2), inhibit phospholipase A₂ (PLA₂). This prevents the release from phospholipid of arachidonic acid, the common precursor of both prostaglandin and leukotriene inflammatory

mediators. Although lipocortins are produced by human cells (3,4) and human lipocortin genes have been cloned (5), the exact role of these proteins in human health and disease has yet to be understood.

As yet, there are no published data on lipocortin in the human lung, whether healthy or abnormal, although lipocortins are present in rat (6) and bovine (7) lungs. However, there is evidence that human lung cells can produce steroid-inducible inhibitors of arachidonic acid release (8), one of which may be a lipocortin.

A previously published study has shown that lipocortin 1 (Lc 1; 37 kDa), the most widely distributed and best characterized member of the lipocortin family, can be proteolyzed *in vitro* by an elastase (probably porcine pancreatic elastase) (9) to a number of products, with molecular weights ranging from 18 to 33 kDa. The functional significance of one such fragment (33 kDa) is of particular interest. Unpublished studies (Flower et al.) show that *in vivo*, the 33-kDa form loses the ability to suppress inflammation; furthermore, it fails to reduce prostaglandin production and release by peritoneal

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macrophages maintained *in vitro*, although it appears to retain 90% of its PLA₂ inhibitory capacity in one cell-free *in vitro* assay system (9). In addition, when the 37-kDa protein is proteolyzed, as well as the 33-kDa form, a 3-kDa fragment is liberated from the N-terminal portion of the parent molecule. There is some evidence that peptides with sequence homology to this 3-kDa fragment may have some inherent anti-inflammatory activity. For example, similar peptides are able to block formyl-methionyl-leucyl-phenylalanine (fmlp)-induced chemotaxis in polymorphonuclear neutrophils (PMN) (10), although these effects occur at high concentrations that are probably nonphysiological. It therefore seems likely that proteolysis of Lc 1 could have significant pathological effects.

Elastase activities in the human lung are greatly increased by tobacco smoking (11); in addition, elevated elastase activities have been implicated in the etiology of many pulmonary diseases including smoking-related disorders and some environmentally induced diseases (12), as well as certain congenital diseases (13).

The first aim of the present study was to establish whether or not Lc 1 is present in the human lung. To do this, lung lavage fluids from healthy human subjects and from patients with various lung diseases were analyzed for Lc 1 and for its breakdown products. The second aim of the current investigation was to determine whether or not Lc 1 was degraded (inactivated?) by purified human neutrophil elastase (NE) and, if so, to find out if the actions of NE on lipocortin could be reproduced by exposure of recombinant Lc 1 to media in which elastase-secreting cells (PMN, and alveolar macrophages, AM) had been cultured.

Methods

Materials

Double-lumened, balloon-tipped catheters (5F and 6F) were obtained from Kimal Scientific Products Ltd. (Uxbridge, Middx., UK). Centricon units were purchased from Amicon Ltd. (Stonehouse, Glos., UK). The PhastSystem electrophoresis apparatus and SDS-polyacrylamide gradient Phastgels to use on it were obtained from Pharmacia Ltd. (Milton Keynes, Bucks, UK), while the dot-blotting apparatus and nitrocellulose paper were purchased from Bio-Rad Laboratories Ltd. (Hemel Hempstead, Herts., UK). The Chromoscan 3 gel scanner was from Joyce-Loebl (Gateshead, Tyne and Wear, UK). All tissue culture media were obtained from Gibco Ltd. (Paisley, Renfrewshire, UK). Rainbow molecular weight markers were purchased from Amersham International plc (Amersham, Bucks, UK). Goat anti-rabbit IgG conjugated to horseradish peroxidase was obtained from Sigma Chemical Co. Ltd. (Poole, Dorset, UK). Neutrophil elastase and its substrate, insoluble elastin, were ordered from Elastin Products Ltd. (Pacific, MO). Recombinant human Lc 1 and monospecific antibodies to the whole molecule were the gifts of Biogen Inc.

(Cambridge, MA). All other chemicals were of AnalaR grade and were purchased from BDH Ltd. (Poole, Dorset, UK).

Subjects

Peripheral lung lavage fluid (PLF;14) was collected from 6 volunteers (1 male; 6 smokers; median age 26 years, range 22–31 years) who underwent bronchoscopy solely for research purposes. Informed consent was obtained from all subjects by a clinician who was not involved in the study, and approval was obtained from the Local Ethical Committee of Charing Cross Hospital.

In addition, PLF was obtained during routine fibre-optic bronchoscopy of 11 patients (5 male; 4 nonsmokers, 4 exsmokers, and 3 current smokers; median age 68 yrs, range 56–78 years). The final diagnoses on these patients were: fibrosing alveolitis, $n = 3$; pleural effusion, $n = 2$; mesothelioma, bronchial carcinoma, and hemoptysis of unknown origin, each $n = 1$; no final diagnosis, $n = 3$.

Central lung lavage fluid (CLF) (14) was collected from 4 male patients (1 nonsmoker, 3 current smokers; median age 61 years, range 57–69 years); and the final diagnoses: bronchial carcinoma, resolving pneumonia, hemoptysis of unknown origin, and no final diagnosis, each $n = 1$.

An additional 22 patients (14 male; 8 nonsmokers, 4 exsmokers, and 10 current smokers; median age 56 years, range 25–75 years) underwent bronchoscopy with bronchoalveolar lavage (BAL) as a routine part of their diagnostic work-up. The final diagnoses on these patients were: normal lung, $n = 5$; bronchial carcinoma, $n = 5$; fibrosing alveolitis, $n = 3$; sarcoidosis, $n = 3$; pulmonary effusion, $n = 2$; aspergillosis, mesothelioma, bronchiectasis, infection, no final diagnosis, each $n = 1$. One subject had mesothelioma with fibrosing alveolitis, and therefore there are $n = 23$ diagnoses. The subjects found to have normal lungs were investigated for unexplained cough, hemoptysis, or cancerphobia.

No subject included in this study was undergoing treatment with glucocorticoids at the time of bronchoscopy.

Collection and Processing of Lavage Fluids and Preparation for Western Blotting

PLF was collected as previously described (14). A 6F double-lumened, balloon-tipped catheter was passed through the biopsy channel of an unwedged BF₁ T Olympus bronchoscope and wedged by inflation of the balloon at the level of the seventh or eighth generation. A 20-mL portion of sterile, warmed 0.15 mole/L saline was instilled through the second lumen and into the lung, below the level of the wedged balloon. It was aspirated immediately into a sterile trap, and the process was repeated until 100 mL saline had been instilled into the lung and aspirated into the same trap.

CLF was collected as previously described (14). Briefly, a 5F double-lumened, balloon-tipped catheter was passed through the bronchoscope as previously described and saline was instilled into the airways—this time above

the level of the balloon, at the fifth or sixth generation. Sterile, warmed 0.15 mole/L saline was instilled in 4 mL portions and aspirated as previously described, until a total of 20 mL had been instilled into the airway and collected into a sterile trap.

BAL was carried out as previously described (15). Warmed, sterile 0.15 mole/L saline (50 mL) was instilled through the biopsy channel of the bronchoscope that was wedged at the third or fourth generation. The BAL fluid (BALF) was withdrawn immediately into a sterile trap and the process repeated a further three times.

All PLF, CLF, and 12 of the BALF were processed in the same way. A sample of 1 to 2 mL was removed for a total cell count using a Neubauer hemocytometer and for a differential cell count following Wright-Geimsa staining. The remaining fluid was filtered through wire mesh and centrifuged at 300g at 4°C for 15 min to pellet the cells. (Cells from two BALF samples were used for the culture studies described in the following section). Lavage supernatants were stored in fractions at -20°C or lower until analysis.

Total protein was measured by the method of Lowry et al. (16) using bovine serum albumin as a standard. Samples (2 mL) of BALF and PLF were concentrated approximately 15-fold and 0.2 mL samples of CLF were concentrated approximately 10-fold by centrifugation in Centricon units, with molecular weight cut-off 10 kDa, for 3 hr at 3800g. The final protein concentration was adjusted to 2.5 mg/mL with 0.15 mole/L saline. Samples were boiled for 5 min with equal volumes of sample buffer, pH 8, containing Tris-HCl (10 mmole/L), EDTA (1 mmole/L), 5% (w/v) SDS, 10% (v/v) mercaptoethanol and 0.02% (w/v) bromophenol blue. The reduced samples were stored at 20°C prior to Western blotting.

Culture of BALF Cells and Incubation of Cell Secretions with Recombinant Human Lipocortin

The cells from 12 BALF samples were cultured as follows: BALF cells and supernatants were separated as previously described. The cells were washed in Hanks' buffered salt solution (without Ca^{2+} or Mg^{2+}), then pelleted by repeat centrifugation at 300g for 15 min at 4°C. The cells were then suspended in Dulbecco's modified Eagle's medium or low-protein hybridoma medium and plated out at a concentration of $10^6/\text{mL}$. After a 1-hr adherence period, the conditioned media and floating cells were removed and separated by centrifugation at 300g at 4°C for 15 min. The media (CM 1) were stored at -20°C. The adherent cells, mainly AM, were cultured in fresh medium for a further 3 hr. Following that, the conditioned media (CM 3) were removed, separated from any residual nonadherent cells as previously described, and stored as previously described.

Fractions of CM 1 and CM 3 were concentrated by lyophilization or by centrifugation in Centricon units,

(cut-off, 10 kDa), at 3800g for 3 hr at 4°C. Pure recombinant Lc 1 (80 $\mu\text{g}/\text{mL}$) was incubated at 37°C with concentrated fractions of CM 1 and CM 3 from the equivalent of 2 to 20×10^6 elastase-secreting cells (PMN and AM)/mL for periods of up to 48 hr. Fractions of the incubation mixtures were removed at various time points, and the reaction was terminated by boiling for 5 min with an equal volume of sample buffer prepared as described earlier. Because the amounts of CM varied between patients, samples were not taken from every subject at every time point. Collections were staggered so that sufficient samples were available to carry out statistical analysis on each time point. In order to determine whether the BALF cells had released any Lc 1 into the medium, samples of CM, to which no exogenous recombinant Lc 1 had been added, were prepared for Western blotting as previously described. Prepared samples were stored at -20°C until analysis.

Western Blotting for Lipocortin 1

SDS-polyacrylamide gel electrophoresis was performed on 8 to 25% gradient Phastgels using a PhastSystem electrophoresis apparatus according to the manufacturer's instructions. A 1- μL fraction of lavage fluid, prepared as previously described, was applied to each gel and electrophoresed for approximately 30 min at 10 mAmps per gel, maximum voltage, 100 V. Pure recombinant human Lc 1 (80 $\mu\text{g}/\text{mL}$) was prepared as described above and used as a control on every gel. Molecular weight markers (14.3–200 kDa) of variously dyed proteins were used on each gel. Samples of recombinant Lc 1 (80 $\mu\text{g}/\text{mL}$) which had been incubated with CM, as previously described, were electrophoresed in the same way.

Protein bands were transferred from gels to nitrocellulose paper by diffusion under pressure for 18 hr at room temperature using Tris-glycine-methanol buffer, pH 8 (Tris 6.25 mmole/L + glycine 250 mmole/L: methanol, 4:1), as a carrier. Completion of protein transfer was monitored by observing the colored bands of rainbow markers.

The nitrocellulose sheets were agitated in Tris-buffered saline (TBS; 0.15 mole/L NaCl, 10 mmole/L Tris-HCl, pH 7), containing 3% (w/v) bovine serum albumin for 1 hr at room temperature in order to block any remaining protein binding sites. The sheets were then shaken gently for 24 hr at 4°C (lavage supernatants), or 2 hr at room temperature (CM/Lc 1 mixtures) with a specific rabbit anti-recombinant human Lc 1 antibody diluted 1 in 1000 with washing buffer [WB; 10 mmole/L Tris-HCl, pH 7, 0.3 mole/L NaCl, 0.5% (v/v) Nonidet P-40] containing 3% (w/v) bovine serum albumin. The remaining steps were performed at room temperature. Nonspecifically bound antibody was removed by exhaustive washing in WB (without serum albumin). Following this, the blot was exposed for 1 to 2 hr to goat anti-rabbit IgG, conjugated to horseradish peroxidase diluted 1 in 1000 in WB, containing 3% (w/v) bovine serum albumin. After further washing in WB followed by TBS, the Lc 1 bands were visualized by incubation of the blots in TBS containing

diaminobenzidine, 125 $\mu\text{g/mL}$ and H_2O_2 , 0.04 $\mu\text{L/mL}$. After a 30- to 60-min incubation, the reaction was terminated by washing the blot in TBS.

Any lavage supernatants that were negative for Lc 1 on Western blotting were immunoblotted using larger amounts of sample. Briefly, 50- μL portions of concentrated, reduced lavage fluid were applied directly to nitrocellulose paper using a dot-blotting apparatus according to the manufacturer's instructions. Any Lc 1 in the sample was then visualized using the antisera and detection system described previously.

Identification of the Partially Degraded Lc 1 Band

Duplicate samples of pure recombinant Lc 1 and Lc 1 partially degraded by incubation with a sample of CM 1 were electrophoresed as previously described on a single gel. The gel was then bisected and one set of samples Western blotted as described above. The second set of samples was blotted using a first antibody raised to a peptide with an identical amino-acid sequence to residues 13 to 26 of human Lc 1. This region of the *N*-terminal portion of Lc 1 contains the tyrosine phosphorylation site situated at position 21 (5).

The antibody was diluted 1 in 100 with WB containing 3% (w/v) bovine serum albumin and was left bathing the nitrocellulose sheet for 24 hr. The rest of the blotting procedure was as previously described, except that the horseradish peroxidase-conjugated second antibody was diluted 1 in 500, not 1 in 1000, and was left in contact with the blot for 3 hr.

Blot Scanning: Use and Validation

The Lc bands detected on each individual track of every blot were scanned using a Chromoscan 3 gel scanner. Each scan was 0.3 mm wide. A scan length of 5 mm was long enough to include the bands of native and degraded Lc 1. The scanner was set in reflectance mode to measure optical density at 530 nm. The machine defined the limits of each band according to preprogrammed parameters, and it calculated the total and relative densities of all the bands within a 5 mm scan. It could therefore calculate the percentage of the total Lc 1 detected in the parent (37 kDa) and proteolyzed (34 kDa) forms.

The between-blot reproducibilities of the absolute and relative densities of the 37 and 34 kDa Lc 1 bands were determined by pooling the control data from every Western blot and calculating the coefficient of variance of these parameters. Western blots with identical samples of pure Lc 1 on every track or with a different amount of pure Lc 1 (0–500 ng) on every track were analyzed in the same way to determine within-blot reproducibility and to determine whether optical density was linear with respect to the amount of Lc 1 applied to the gel.

Effect of Human Neutrophil Elastase on Recombinant Human Lc 1

Human neutrophil elastase (NE) was active site titrated using constants taken from Nakajima et al. (17). Recombinant

human Lc 1 was incubated with active (NE) at 37°C in low protein hybridoma medium in molar ratios of 0.01 to 1 (NE) to 1 (Lc 1). Fractions of incubation mixtures were removed at intervals up to 48 hr. The reaction was terminated and the samples analyzed by Western blotting as previously described.

Determination of Elastase Activity (EA)

Elastase activities (EA) of samples of CM and BALF, and PLF supernatants were measured against insoluble elastin (from bovine ligamentum nuchae) tritiated as previously described (18). EA was assayed by the method of Banda and Werb (18), modified as previously described (15). Human NE active site, titrated as earlier described, was used as a standard.

Statistics

The Mann-Whitney *U* test was used to compare lavage data, while the Wilcoxon signed rank test for paired data was used to analyze the effect of CM on Lc 1. The Spearman rank correlation coefficient, corrected for tied values, was calculated to assess the relationship between variables. In all analyses, $p < 0.05$ in a two-tailed test was taken as statistically significant.

Results

Validation of Scanning Technique

There are two ways of analyzing the degradation of native Lc 1 (i.e., from 37 to 34 kDa) from a Western blot scan. The first involves measuring the reduction in optical density of the 37 kDa band. This method requires a linear relationship between optical density and the amount of protein in the band. However, the relationship was nonlinear, particularly at high concentrations of Lc 1. Furthermore, when equal amounts of exogenous Lc 1 were analyzed on each track, the reproducibility of total optical density measurements was very poor (Table 1). The alternative method of estimating degradation is to measure the total density of the 37 kDa (parent) band plus that of the 34 kDa (degraded) band and calculate the proportion of the total density contributed by each band. Using this method, it was found that the contribution of the 37 kDa band to the total optical density was very consistent within and between blots (Table 1) and irrespective of the total amount of Lc 1 applied to the gel. Therefore, this measurement has been used throughout the study.

Comparative Analysis of BALF, PLF, and CLF

Lipocortin. LC 1 was detected by Western blotting in PLF from all healthy volunteers and in 8 of the 11 PLF samples from patients. In addition, Lc 1 was detected in 11 of the 12 BALF samples tested, but it was not detected in CLF from any subject. However, when samples of negatively staining lavage fluids were dot-

Table 1. Validation of scanning technique.

Parameter	Condition	n	$\bar{x} \pm \text{SD}^a$	Coefficient of variance
Total density ^b	Within blot	7	3144 \pm 1006	32%
Total density	Between blot	45	2240 \pm 1822	81%
37 kDa, % of total	Within blot	14	91 \pm 3	2%
37 kDa, % of total	Between blot	22	93 \pm 5	5%

^aSD, standard deviation.

^bTotal density is measured in arbitrary units.

blotted, Lc 1 was then detected in all CLF and PLF and in all but one of the BALF (data not shown).

The proportions of native (37 kDa) to degraded (34 kDa) Lc 1 varied considerably between subjects. However, PLF from volunteers contained at least 70% Lc 1 in the native form (Fig. 1), which was significantly higher than in PLF from patients (Table 2). In contrast, in BALF, the proportion of 37 to 34 kDa Lc 1 was more variable and there was no statistically significant difference in the percentage of Lc 1 in the native form between BALF from patients with normal and abnormal lungs (Table 2).

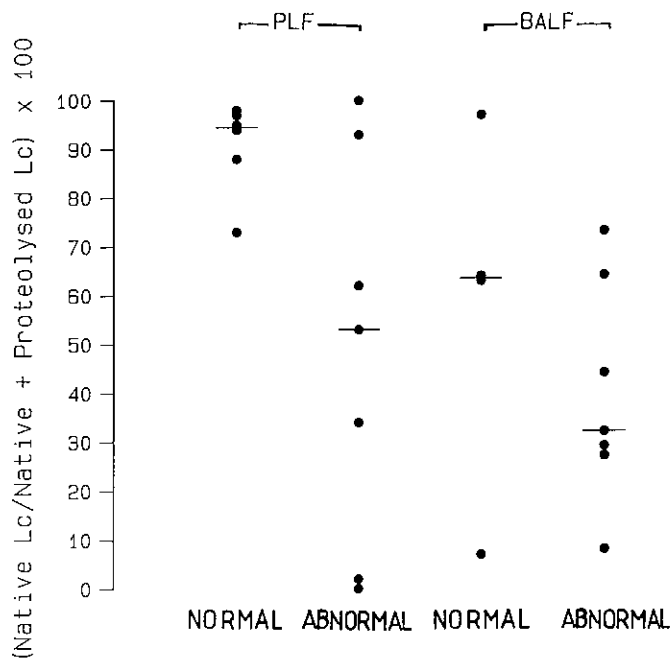


FIGURE 1: Percentage of 37 kDa Lc 1 present in PLF and BALF from subjects with normal and abnormal lungs. See text and Table 2 for statistically significant differences between groups and for correlations with other lavage parameters.

Table 2. Recovery of fluid and cells, protein levels, elastase activities and proportions of native Lc 1 in different types of lavage fluid from subjects with normal and abnormal lungs.

Group	n	Percent of fluid recovered	Cells, $\times 10^6$	Cell profile, % total	Total protein, mg	EA, ng/mg protein	37 kDa Lc 1, % total ^a
Normal/volunteer PLF	6	33(21-40)	16.3(12.2-21.3)	AM 94(81-100) PMN 4(0-14) LYM 4(0-7)	7.33(5.25-17.55)	27(3-103)	95(73-98)
Abnormal PLF	11	31(10-68)	4.7(0.1-9.6) ^b	AM 87(58-99) PMN 2(1-25) LYM 8(0-24)	1.88(0.39-14.15) ^b	36(0-559)	53(0-100) ^b
Normal patient BALF	5	67(31-75) ^c	27.5(5.4-108.8)	AM 91(80-96) PMM 1(0-5) LYM 4(3-20)	15.88(5.59-47.85)	175(72-395) ^c	63(0-97) ^b
Abnormal BALF	7	39(18-58)	15.3(4.1-58.0) ^d	AM 55(45-79) ^e PMN 35(7-56) ^f LYM 11(1-15)	18.50(7.56-41.70) ^d	255(38-2860)	32(8-73)
Abnormal CLF	4	33(15-45)	0(0-0) ^g	AM ND PMN ND LYM ND	0.39(0.02-0.73) ^g	NA	ND

^a Only samples positive for Lc 1 on Western blotting are included; see Figure 1.

^b < Normal PLF.

^c > Normal PLF.

^d > Abnormal PLF.

^e < Abnormal PLF and normal BALF.

^f > Abnormal PLF and normal BALF.

^g < Abnormal PLF and abnormal BALF.

When PLF and BALF were compared to each other, it was found that PLF from volunteers had a significantly greater percentage of Lc 1 in its native form than BALF from patients with normal lungs (Table 2).

Other Variables. Although there was no statistically significant difference in the percentage of instilled fluid recovered in PLF from volunteers compared to that from patients, more protein was recovered from volunteer's PLF (Table 2). More cells were recovered from volunteer's PLF, but the cell profiles were not significantly different to those from patients. EA/mg protein did not differ between the groups and neither did EA/lavage (data not shown), despite the significant difference in protein recoveries.

There were no significant differences in fluid recoveries, levels of total protein, or numbers of BALF cells in BALF from patients with normal lungs compared to those with abnormal lungs (Table 2). However, a significantly greater proportion of the recovered cells in abnormal BALF were PMN (Table 2). EA/mg protein did not differ between the two sets of BALF.

When BALF and PLF from subjects with healthy lungs were compared, a greater percentage of the instilled fluid was recovered after BALF, but the total protein, number, and type of cells recovered was not significantly different between the lavage types. However, the EA/mg protein was significantly greater in BALF than in PLF (Table 2).

Comparison of BALF and PLF from subjects with abnormal lungs showed no difference in the percentage of instilled fluid recovered. However, protein levels were higher in BALF than in PLF. Significantly more cells were recovered in BALF from patients with abnormal lungs, and the proportion of PMN was greater than in abnormal PLF (Table 2). EA/mg protein tended to be greater in abnormal BALF than in PLF from patients with abnormal lungs, but this difference was not statistically significant.

The percentage of instilled fluid recovered after CLF was not significantly different to that after other types of lavage. Cells were not detected in any of the CLF, and protein levels were significantly lower than in other lavage types (Table 2). EA was not assayed in these samples, because of the small volumes of material available.

Correlations of Lipocortin with Other Variables. No statistically significant correlations were found between the proportion of Lc 1 in the 37-kDa form and any variable in PLF from patients or volunteers. However, in BALF from patients with abnormal lungs, there was an inverse correlation between the proportion of Lc 1 remaining in the native (37 kDa) form and the number of PMN in the lavage ($n = 7$ pairs, $rs = -0.8214$, $p < 0.05$). In addition, there was a tendency towards an inverse correlation between the percentage of native (37 kDa) Lc 1 and EA/mg protein ($n = 7$ pairs, $rs = -0.7500$, $p < 0.10$) in BALF from patients with abnormal lungs. No such relationships were observed in BALF from patients with apparently normal lungs.

Analysis of BALF Cell Secretions in CM 1 and CM 3 and Their Effect on Recombinant Lc 1

Under the conditions employed in this study, Lc 1 could not be detected in any sample of CM before the addition of exogenous recombinant protein.

The cellular equivalents of the CM 1 and CM 3 incubated with recombinant Lc 1 are shown in Table 3. PMN were present in CM 1, but not CM 3. EA/ 10^5 elastase secreting cells (ESC) was higher in CM 1 than in CM 3 (CM 1, 3.3 [0.5–10.0 ng/ 10^5 cells]; CM 3, 0.5 [0–9.7 ng/ 10^5 cells] median [range] CM 3 < CM 1, $p < 0.05$). Therefore, because for any given subject, Lc 1 was incubated with CM 1 and CM 3 derived from equivalent numbers of elastase secreting cells, the EA/Lc 1 ratio was significantly higher when Lc 1 was incubated with CM 1 than CM 3 (Table 3). There were no significant correlations between EA and PMN or AM number in CM 1 or CM 3.

All but two of the CM 1 tested were able to degrade recombinant Lc 1 (Table 4), although some samples were much more active in this respect than others. CM 3 were generally less active than CM 1 (Fig. 2), although the groups were not statistically significantly different to each other, because of the variability between different CM 1 samples in the capacity to degrade Lc 1.

In CM 1 the disappearance of parent (37 kDa) Lc 1 was positively correlated to the PMN/Lc 1 ratio in the samples after 1 and 6 hr incubations, and it tended to be correlated after 24 hr (Table 4; 1 hr, $n = 10$ pairs, $rs = -0.7200$, $p < 0.05$; 6 hr, $n = 6$ pairs, $rs = -0.9241$, $p < 0.05$; 24 hr, $n = 10$ pairs, $rs = -0.6398$, $p < 0.10$). In contrast, the number of AM was inversely correlated with the disappearance of 37-kDa form of Lc 1 at the 6-hr time point (Table 4; $n = 6$ pairs, $rs = -0.6891$, $p < 0.05$). The disappearance of parent Lc 1 was not significantly correlated to EA/Lc 1 ratio in CM 1 at any time point. There were no statistically significant correlations between the percentage of native Lc 1 remaining and any of the parameters measured in CM 3.

Identification of the Partially Degraded Form of Lc 1

Although the antibody raised to an amino-acid sequence in the N-terminal portion of native Lc 1 cross-reacted with the 37-kDa protein, it failed to cross-react with the 34 kDa form produced following incubation for 3 hr with CM 1 from a mixed population of cells (Fig. 3).

Effect of Pure Human NE on Recombinant Lc 1

Incubation of pure NE and Lc 1 at a molar ratio of 0.1:1 or 0.5:1 resulted in complete degradation of the 37-kDa protein to the 34-kDa form within 0.5 hr, while at a ratio of 1:1, no lipocortin-derived protein of any molecular weight could be detected on a Western blot following a

Table 3. Cell profile from which CM 1 and CM 3 were derived and the effect of these cells on pure recombinant Lc 1.

Parameter	Ratio/Lc 1 CM 1 <i>n</i> > 10	Ratio/Lc 1 CM 3 <i>n</i> > 9	Control medium <i>n</i> = 2
ESC, $\times 10^5$ ^a	1.0(0.4-2.0)	1.0(0.4-1.0)	0
AM, $\times 10^5$	0.7(0.3-1.0) ^b	1.0(0.4-1.0) ^c	0
PMN, $\times 10^5$	0.1(0-0.5) ^d	0(0-0) ^e	0
EA, NEE ng ^f	3.0(0.5-9.1)	0.3(0-9.7) ^e	0

Table 4. Time course of Lc 1 degradation by CM 1 and CM 3.

Time of incubation, hr	37 kDa, % remaining CM 1 <i>n</i> > 6	37 kDa, % remaining CM 3 <i>n</i> = 6	Control medium <i>n</i> = 3
0	100	100	100
0.5	94(29-108) ^g	—	102
1.0	91(0-101) ^g	101(55-110)	100
3.0	81(0-102) ^g	100(0-114)	99
6.0	42(0-97) ^g	101(0-105)	102
18.0	4(0-105) ^g	101(0-114)	—
24.0	32(0-105) ^g	97(0-99) ^g	101
48.0	0(0-105) ^g	86(0-101)	100

^aElastase secreting cells.

^b+ve correlation with 37 kDa remaining in CM 1, 6 hr.

^c> CM 1.

^d-ve correlation with percentage of percentage of 37 kDa remaining in CM 1, 1 hr and 6 hr.

^e< CM 1.

^fneutrophil elastase equivalents.

^g< time 0 hr.

See text for *rs* values. *p* < 0.05 for all data.

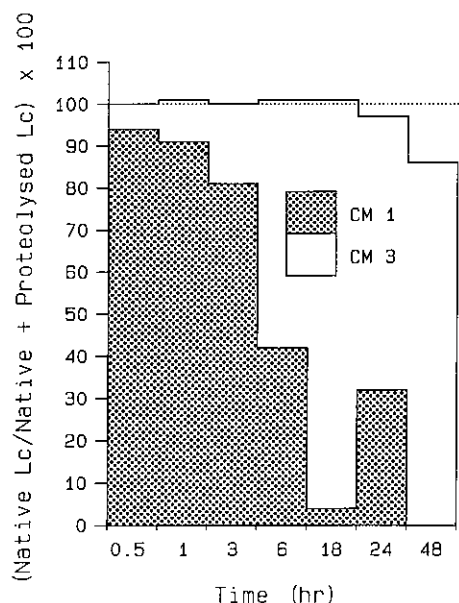


FIGURE 2. Median percentage of 37 kDa human recombinant Lc 1 remaining after different times of incubation with samples of conditioned media (CM 1 and CM 3). CM 1: Percentage of 37 kDa remaining < time 0 hr at all incubations times of 1 hr or more, *p* < 0.05. CM 3: Percentage of 37 kDa remaining < time 0 hr only after 24 hr incubation, *p* < 0.05.

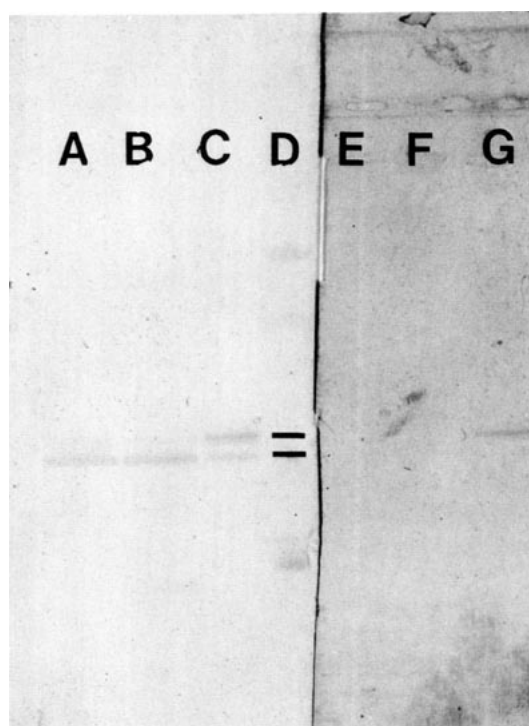


FIGURE 3: Comparison of Western blots of pure recombinant human Lc 1 using antibodies raised to the whole Lc 1 molecule (tracks A-D) or to a peptide consisting of residues 13 to 26 from the *N*-terminal portion of the molecule (tracks E-G). Track D contains molecular weight markers. The upper horizontal line corresponds to a molecular weight of 37 kDa, the lower to a weight of 34 kDa. Tracks C and G contain untreated pure recombinant human Lc 1. A small proportion is in the 34-kDa form, but only the parent protein is detected by antibodies to the *N*-terminal sequence. Tracks A, B, E, and F contain recombinant human Lc 1 pretreated for 3 hr with a sample of CM 1. Tracks A and B show that the majority of the Lc 1 has a molecular weight of 34 kDa. No bands are visible on tracks E and F, demonstrating that the 34 kDa portion of the molecule lacks the *N*-terminal sequence.

0.5 hr incubation (Fig. 4). Decreasing the NE:Lc 1 ratio to 0.01:1 reduced the rate of loss of lipocortin-derived protein. However, even at this low ratio, all the protein was in the 34-kDa form by 0.5 hr. After a 24-hr incubation with NE at a ratio of 0.01:1 (NE:Lc 1), no lipocortin-derived protein was detectable on a Western blot (Fig. 4).

Discussion

Lipocortin 1 was detected in all but one of the lavage samples analyzed, irrespective of the clinical status or smoking history of the subjects. We believe this to be the first report of the presence of any member of the lipocortin family in human lung lavage fluid. In addition, it has been shown that, although in healthy volunteers the Lc 1 was predominantly in its native form, in many of the lavage samples from patients, a high proportion of the protein was partially degraded. Our *in vitro* studies suggest that this degradation could be caused by one or more

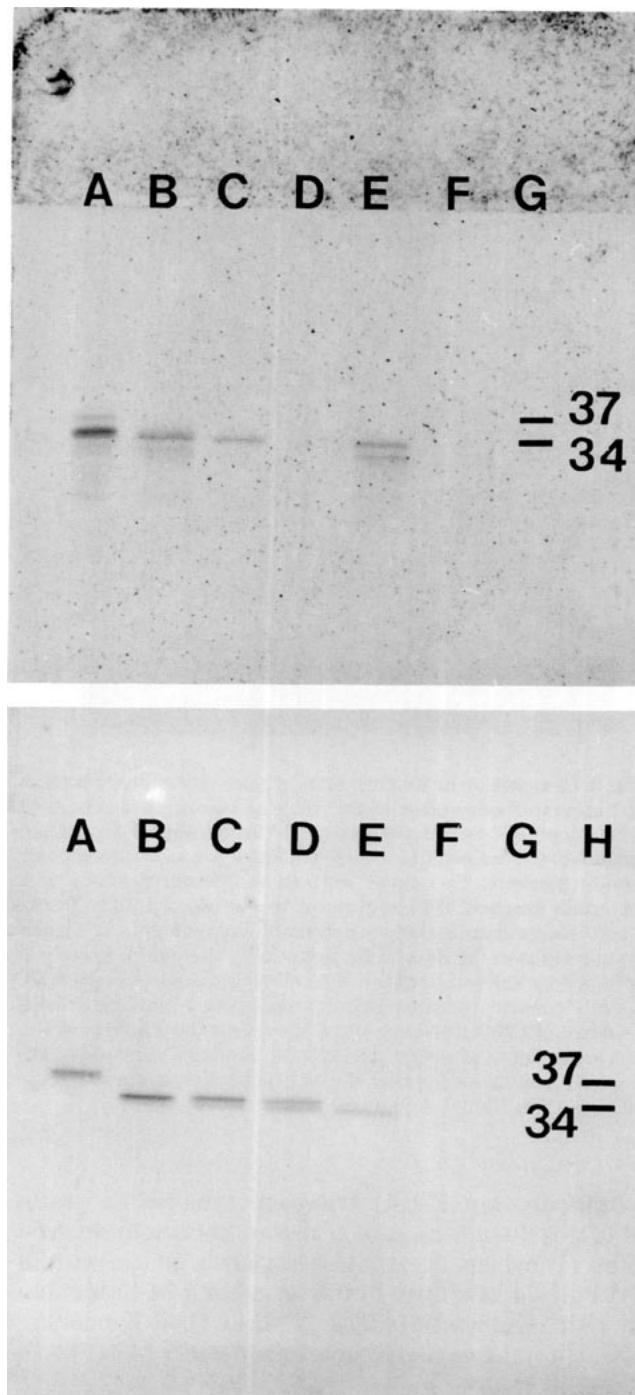


FIGURE 4. Western blot of recombinant human Lc 1 following preincubation with NE at different molar ratios. (A) Track G contains molecular weight markers. Tracks A–D contain Lc 1 pretreated with NE at a ratio of 0.5:1 (NE:Lc 1) for 1, 10, 30, and 60 min respectively. After a 1-min exposure, the majority of the Lc 1 is partially degraded. By 10 min it is all partially degraded, and by 60 min it is completely proteolyzed. Tracks E and F contain Lc 1 pretreated with NE at a ratio of 1:1 for 10 and 30 min, respectively. Following a 10-min exposure, all the Lc 1 is partially degraded, while by 30 min it is completely proteolyzed. (C). Track H contains molecular weight markers. Tracks A–G contain recombinant human Lc 1 pretreated with NE at a molar ratio of 0.01:1 (NE:Lc 1) for 0, 0.5, 1, 3, 6, 24, and 48 hr, respectively. Within 30 min all the Lc 1 has been proteolyzed to the 34 kDa.

products of the PMN, while studies with a specific antibody indicate that this partially degraded Lc 1 lacks the *N*-terminal portion that we believe to be essential for full anti-inflammatory function *in vivo* and in cellular systems *in vitro* (Flower et al., unpublished observation).

In previous studies, lipocortins have been detected in lung tissue from the rat (6), from cattle (7) and in feline tracheal rings (19). The most likely source of the Lc 1 detected in our lung lavage fluids is the AM, since studies in the rat (5,20) and on cell lines from various species (6) show that monocytes and macrophages are frequently rich sources of lipocortins. Lipocortins require Ca^{2+} to remain attached to the plasma membrane, and thus, lavage of the lung with a calcium-free solution (NaCl) may result in some of the Lc 1 becoming detached from the AM surface and solubilized in the supernatant fraction of the lavage fluid. However, the possibility that Lc 1 exists in a free form at the epithelial surface cannot be excluded.

In the current study, amounts of Lc 1 could not be quantified. This is probably because factors such as ambient temperature and the activity of conjugated horseradish peroxidase are difficult to control but may modify the density of staining. In contrast, the relative proportion of 37 kDa to proteolyzed (34 kDa) Lc 1 was very reproducible (Table 1) and has, therefore, been determined.

Taking into account the degree of resolution obtained with SDS-polyacrylamide electrophoresis, it is probable that the 34-kDa band observed throughout the current study is the same or very similar to the 33-kDa species generated from human recombinant Lc 1 by Huang et al. (9). These authors showed their fragment to be clipped 30 residues from the *N*-terminal of the precursor (37 kDa) molecule. In addition, an earlier study by the same group (6) demonstrated that some of the endogenous Lc 1 isolated from rat peritoneal extracts was clipped in the same region. Thus, it seems that both *in vivo* and *in vitro*, this region of Lc 1 is the most vulnerable to proteolysis. In the current study, it has been shown that the partially degraded Lc 1 occurs in the human lung (Table 1) and that NE and possibly other products of the PMN are capable of causing such degradation (Table 3). In addition, this study has shown that the 34-kDa form lacks the *N*-terminal end of the parent protein since it does not cross-react with an antibody to this epitope of the molecule (Fig. 3), increasing the probability that we have studied the same molecular species as Huang et al. (9). Although the results of cell-free PLA_2 assays (9) suggest that the clipped form inhibits PLA_2 , other authors consider cell-free systems to be flawed (21). Therefore, because we believe bioassay to be more relevant to the situation in man, we are confident that measurement of the ratio of 37:34-kDa forms is actually a measure of the ratio of functional:nonfunctional Lc 1.

The results of the *in vitro* studies (Table 3) show that the recombinant Lc 1 was much more actively degraded by conditioned media from mixed populations of cells containing AM and PMN than that from adherent AM

alone, suggesting that the degraded Lc 1 observed in lung lavage samples may be the result of PMN activity. This was supported by the observation that degradation of 37 kDa Lc 1 was related to the number of PMN in CM 1 (Table 3). It is not clear from these data what factor (or factors) released into CM1 by the PMN is responsible for the breakdown of Lc 1. Even though pure NE is able to proteolyze Lc 1 (Fig. 3), no relationship was observed between EA and proteolysis of Lc 1 in our *in vitro* experiment. There are a number of possible reasons for this; EA is a measure of activity from all elastolytic enzymes, some of which may not degrade Lc 1, thus in the *in vitro* study, metalloelastase from the AM (22) may have made a significant contribution to the EA measured. Furthermore, it is possible that two or more enzymes act in sequence to degrade Lc 1—for example, studies with the pure protein show that NE first degrades Lc 1 to the 34-kDa form (Fig. 4). This may cause a three-dimensional change to the lipocortin structure that makes it more susceptible to attack by other PMN proteases, for example, cathepsins. Equally, an unidentified protease could make the initial clip in the parent Lc 1 and NE could perform subsequent degradation.

In view of the animal studies showing macrophages to be rich in lipocortins (5,20), it is perhaps surprising that endogenous Lc 1 was not detected in media where AM had been cultured (CM). However, the CM samples were taken from very small numbers of cells and had media from a larger cell population been investigated or the sensitivity of the detection system been increased, Lc 1 might have been detected. Alternatively, because the tissue culture media contain Ca^{2+} , any Lc 1 produced by the AM may have remained attached to the plasma membrane and, thus, would not be detected in CM. Alternatively, Lc 1 released into the CM may have already been proteolyzed to small peptides that would not be detected on a Western blot. A final possibility is that human AM synthesize a member of the lipocortin family other than Lc 1, which would not cross-react with our antibody. Further studies will be necessary to differentiate between these possibilities.

The results of the immunoblotting experiments indicate that some samples of lavage fluid contain more Lc 1 than others. While the reasons for differences between subjects undergoing the same type of lavage are unclear, the low Lc 1 levels in CLF, compared to PLF and BALF, may reflect the paucity of AM in the large airways washed during the central lavage procedure. However, it is also possible that in some lavages, particularly in CLF, which contain relatively high protease activities (14), the Lc 1 was degraded to small peptides that would not be detected on a Western blot.

The lavage data (Table 2) suggest that the proportion of native to partially degraded Lc 1 reflects both the health of the subject and the region of the lung sampled. PLF contains proportionally less 34 kDa Lc1 than BALF. Unlike BALF, PLF contains no material from above the seventh generation, so it is likely that much of the 34 kDa Lc 1 in BALF is from the airways. Analysis of more

concentrated samples of CLF or immunoblotting with the antibody to the N-terminal epitope would clarify this point. It is possible that the mucociliary escalator is a route for clearance, either of nonfunctional lipocortin or of cells (probably AM) carrying nonfunctional lipocortin. Alternatively, more degradation of lipocortin may occur in the upper respiratory tract than in the periphery, a possibility supported by earlier studies from this laboratory showing that protease activity per unit albumin and the number of PMN (expressed as a percentage of the total cell population) are both higher in CLF than in PLF (14). Also, it is known that phosphorylation of the tyrosine residue at position 21 of Lc 1 down-regulates its PLA_2 -inhibitory capacity (23) and that once phosphorylated, it is more susceptible to degradation. Thus, it is possible that in the upper respiratory tract, cells are stimulated (for example, to phagocytize and degrade large inhaled particles deposited in the upper airways), and that the subsequent downregulation of lipocortin by phosphorylation results in an increase in proteolysis of Lc 1.

Summary

In summary, we have demonstrated that Lc 1 is present at the epithelial surface of the human lung, usually in a variable mixture of native and partially degraded forms. This study suggests a role for the PMN in inactivation of this protein that may be important in inflammatory lung diseases. Further studies to bioassay and quantify the Lc 1 will be necessary before the physiological significance of these observations can be fully evaluated.

An influx of PMN during inflammation may be a mechanism of downregulating lipocortins and would permit the inflammatory response necessary to destroy a provocative stimulus. Removal of the stimulus with reduced PMN numbers would halt lipocortin degradation. Equally, it is possible that in chronic inflammation, the inactivation of lipocortin is pathological, perhaps when the stimulus cannot be destroyed, for example, in asbestosis or pneumoconiosis. In such situations a massive increase in lipocortin synthesis, induced by high doses of exogenous glucocorticoids, may then be the only way of halting the inflammatory process.

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